

# Aberrant Gene Expression in Human Non Small Cell Lung Carcinoma Cells Exposed to Demethylating Agent 5-Aza-2'-Deoxycytidine

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## Abstract

The identification of genes undergoing genetic or epigenetic alterations and contributing to the development of cancer is critical to our understanding of the molecular mechanisms of carcinogenesis. A new approach in identifying alterations of genes that might be relevant to the process of tumor development was used in this study by examining the gene expression profile in human lung cancer cells exposed to 5-aza-2'-deoxycytidine (5-aza-dC). A cDNA array analysis was carried out on 5-aza-dC-treated and untreated non small cell lung cancer (NSCLC) cell line NCI-H522. Sixteen and 14 genes were upregulated and downregulated, respectively, by 5-aza-dC treatment. Among them, downregulation of tyrosine protein kinase ABL2 (*ABL2*) gene and upregulation of hint/protein kinase C inhibitor 1 (*Hint/PKCI-1*), *DVL1*, *TIMP-1*, and *TRP-1* genes were found in expanded observations in two or three of five 5-aza-dC-treated NSCLC cell lines. Among these genes, we found that cDNA transfer of *Hint/PKCI-1* resulted in a significant *in vitro* growth inhibition in two cell lines exhibiting 5-aza-dC-induced upregulation of *Hint/PKCI-1* and significantly reduced *in vivo* tumorigenicity of one NSCLC cell line. *Hint/PKCI-1*, which is the only other characterized human histidine triad (HIT) nucleotide-binding protein in addition to tumor-suppressor gene *FHIT*, might be involved in lung carcinogenesis.

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damage induced by the respiratory epithelium to cigarette smoke and/or occupational carcinogens is one of the major mechanisms responsible for the development of lung cancer. Epigenetic damage, consisting mainly of promoter hypermethylation, disrupts or silences the expression of tumor-suppressor genes, leading to uncontrolled cell proliferation. There are an increasing number of candidate tumor-suppressor genes that are inactivated by promoter hypermethylation in various types of cancer. In human cancer, promoter hypermethylation appears to be involved at least as frequently as point mutations in the disruption of tumor-suppressor genes [3]. Promoter hypermethylation in tumor-suppressor genes, such as *p16*, death-associated protein kinase (*DAPK*), *FHIT*, and Ras effector homologue (*RASSF1A*), is thought to be an early event in cigarette smoking-related respiratory carcinogenesis [4–6].

One major challenge in lung cancer investigations is the identification of genes that undergo genetic or epigenetic damage during neoplastic development. The basic strategy employed for identifying new candidate genes was the comparison of gene expression between cancer cells and the corresponding noncancerous cells, or between parental tumor cells and anti-tumor agent-treated tumor cells. The comparison of gene expression between 5-aza-2'-deoxycytidine (5-aza-dC)-treated and parental tumor cell lines is a feasible approach for the identification of differentially expressed cancer-related genes [7,8].

5-Aza-dC is the most commonly used DNA demethylation agent and can induce tumor cell differentiation, cell cycle arrest, and apoptosis [9–13]. It is believed that the multiple effects of 5-aza-dC on tumor cells derive from its ability to inhibit DNA methyltransferase during DNA synthesis, leading to DNA demethylation and subsequent activation of the expression of transcriptionally silenced genes [14–16]. The majority of genes activated by 5-aza-dC, such as *Rb*, *p16*, *E-cadherin*, *APC*, *VHL*, *retinoic acid receptor*  $\beta$ , *BRCA1*, and

## Introduction

Lung cancer, the majority of which are non small cell lung carcinoma (NSCLC), is the leading cause of cancer death in men and women in the United States [1]. Although most lung cancers are related to tobacco use, it is also ranked second only to bladder cancer in the proportion cases thought to be due to occupational exposures [2]. Increasing evidence demonstrates that the accumulation of epigenetic

Abbreviations: NSCLC, non small cell lung carcinoma; 5-aza-dC, 5-aza-2'-deoxycytidine; ABL2, tyrosine protein kinase ABL2; Hint/PKCI-1, hint protein/protein kinase C inhibitor 1. Address all correspondence to: Bao-Zhu Yuan, 1095 Willowdale Road, M/S L-3014, Morgantown, WV 26505. E-mail: bby1@cdc.gov

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*DLC-1*, are tumor-suppressor genes in different types of cancer cells [17–25]. It is known that tumor-suppressor genes can negatively regulate the function of oncogenes or genes promoting tumor cell growth through different signal transduction pathways. Therefore, it is presumed that 5-aza-dC exerts its antitumor function by directly upregulating tumor-suppressor genes through DNA demethylation, and by indirectly downregulating oncogenes through signal transduction pathways. Thus, changes in gene expression, especially the upregulation of tumor-suppressor genes and the downregulation of oncogenes, would be expected in each tumor cell line, which is responsive to 5-aza-dC in cell growth inhibition, cell cycle arrest, or apoptosis, during the transition of DNA methylation status rendered by 5-aza-dC treatment. It can also be understood that the transition of DNA methylation status can provide an opportunity to identify tumor-related genes, either tumor-suppressor gene(s) or oncogene(s).

In this study, we employed a cDNA array to analyze the changes in gene expression in an *in vitro* 5-aza-dC-treated human lung adenocarcinoma cell line. Treatment of this cell line with 5-aza-dC resulted in growth inhibition, cell cycle arrest, apoptosis, and changes in mRNA expression of several genes. Among them, the hint/protein kinase C inhibitor 1 (*Hint/PKCI-1*) gene was upregulated by 5-aza-dC and inhibited lung tumor cells growth.

## Materials and Methods

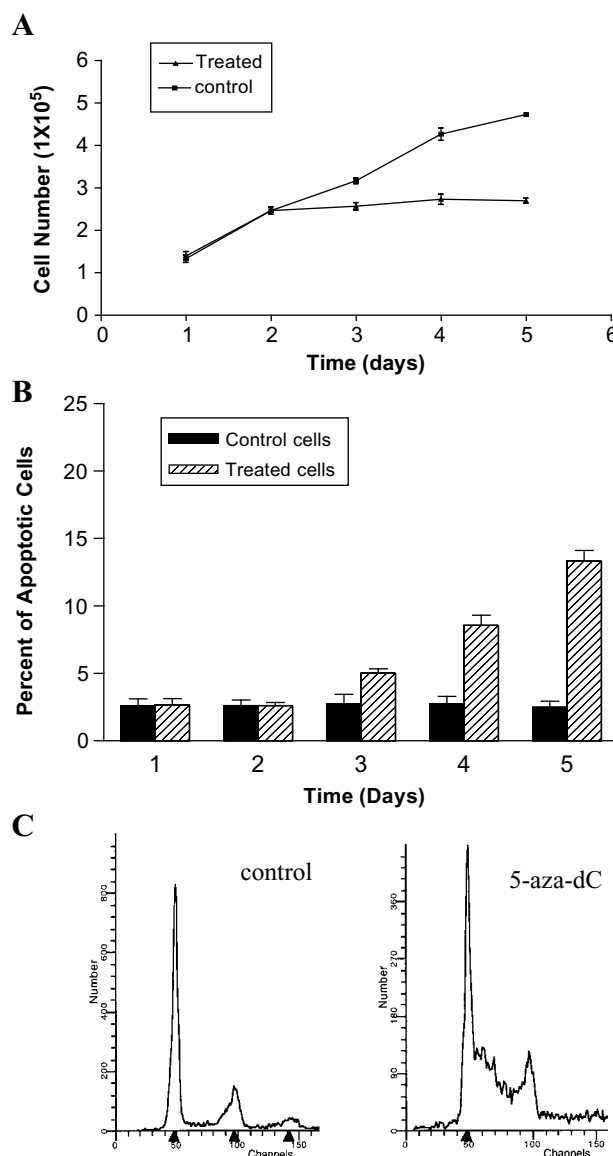
### Reagents and Cell Culture

5-Aza-dC was purchased from Sigma (St. Louis, MO). Atlas Human Cancer 1.2 Array, carrying cDNA fragments of a total of 1176 individual genes, was purchased from Clontech (Palo Alto, CA). *In Situ* Cell Death Detection (TDT-mediated dUTP biotin nick end labeling [TUNEL] assay) Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Human NSCLC cell lines A539, NCI-H23, NCI-H358, NCI-H522, and NCI-H520 were purchased from ATCC (Rockville, MD) and cultured in RPMI 1640 medium (Gibco BRL, Gibco, Carlsbad, CA) containing 10% of fetal bovine serum and 100 U of penicillin and streptomycin.

### Cell Proliferation Assay, TUNEL Assay (In Vitro Cell Death Assay), and Cell Cycle Analysis for 5-Aza-dC-Treated NCI-H522 Cells

In cell proliferation assays,  $1 \times 10^5$  cells were seeded in each T-25 culture flask in triplicate. Cells were either treated or untreated with  $1 \mu\text{M}$  5-aza-dC, and then trypsinized and collected at 24, 48, 72, 96, and 120 hours of treatment. Viable cells determined by trypan blue (Gibco, Carlsbad, CA) exclusion were counted using a hemacytometer.

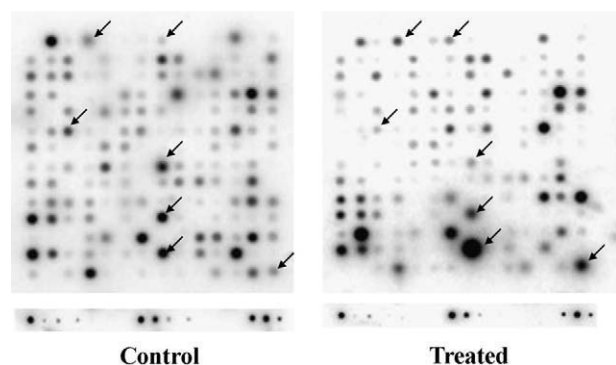
In TUNEL assays, 1 day before treatment, tumor cells either treated or untreated with 5-aza-dC were plated in four-well chamber slides. The cells were fixed at each time point of 24, 72, 96, and 120 hours of treatment by 2% paraformaldehyde solution (in phosphate-buffered saline [PBS], pH 7.4) for 60 minutes at room temperature, permeated in 0.1%



**Figure 1.** Cell proliferation assay, TUNEL assay, and cell cycle analysis for NCI-H522 cells treated with  $1 \mu\text{M}$  5-aza-dC. Cell proliferation assay (A) demonstrated that cells started slower growth at the second day of 5-aza-dC treatment. TUNEL assay (B) showed that about 13% of cells becomes apoptotic in drug-treated cells compared with 2% of apoptotic cells in untreated cells. Cell cycle analysis (C) indicated that 5-aza-dC treatment can cause a significant S-phase arrest in the cell cycle.

Triton X-100/0.1% sodium acetate for 2 minutes on ice, and then labeled with TUNEL reaction mixture containing calf thymus DNA terminal transferase and fluorescein-labeled dNTP at  $37^\circ\text{C}$  for 1 hour. After applying antifade and mounting medium on the slide, fluorescein-labeled cells were detected by fluorescence microscopy and the ratio of the number of labeled cells *versus* the number of total cells was obtained by counting the cells of 10 observation fields.

In the cell cycle analysis, both  $1 \mu\text{M}$  5-aza-dC-treated and untreated cells were collected at 24, 48, 72, 96, and 120 hours of treatment in PBS buffer containing 10 mM glucose and then fixed in 70% ethanol at  $4^\circ\text{C}$  for at least 1 hours. The cells were then stained for 30 minutes in propidium iodide



**Figure 2.** A representative cDNA array analysis of NCI-H522 cells. The changes in gene expression, either downregulation or upregulation, after 4 days of 1  $\mu$ M 5-aza-dC treatment are indicated by arrows.

solution containing 7.5 mM propidium iodide, 10 mM glucose, and 100 U/ml RNase A in PBS buffer. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), which was equipped with Cell Quest version 3.1f software (Becton Dickinson Biosciences, San Jose, CA) for cell cycle data collection. Cell cycle distribution was analyzed using ModFit LT Version 2.0 software (Verity Software House, Inc., Topsham, ME).

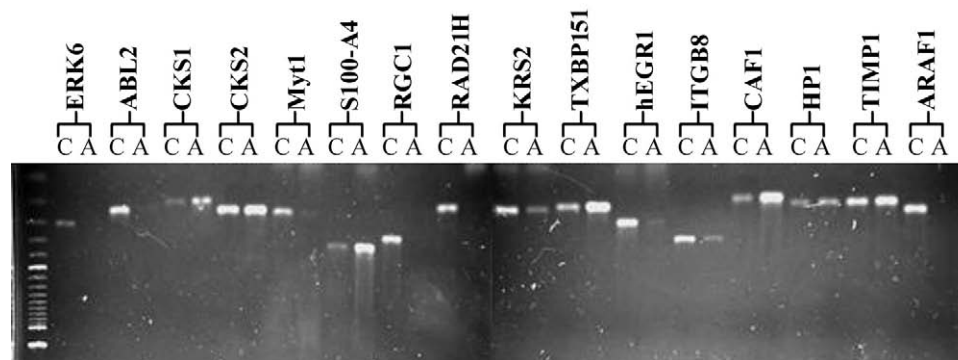
#### cDNA Expression Array Analysis and Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Confirmation for Gene Expression

Polyadenylated RNA from 5-aza-dC–treated NCI-H522 and untreated control cells was extracted with TriZol reagent (Life Technologies, Inc., Grand Island, NY) and purified with magnetic oligo(dT) beads (DYNAL, Inc., Lake Success, NY). In each cDNA array analysis, 0.6  $\mu$ g of mRNA was radio-labeled with a mixture of gene-specific primers during the reverse transcription procedure. The labeled probe of a total of  $7.5 \times 10^6$  cpm activity was hybridized with each array membrane. Membrane washing was performed by following the procedure described in the user's manual. The hybridized membrane was exposed to a PhosphorImage plate and processed using Software ImageQuant Version 3.3 (Molec-

ular Dynamics, Sunnyval, CA). The quantitative data analysis was performed using AtlasImage 2.0 Software (cat no. V1212-1; Clontech). For verifying the alterations of gene expression detected by cDNA array analysis, total full-length cDNA of both treated and untreated cells were synthesized by reverse transcription and amplified by the SMART PCR cDNA Synthesis Kit in a low cycle number (BD Biosciences, Palo Alto, CA). All cDNA samples were diluted into equal concentration according to the PCR production of *GAPDH* before use in the comparison of gene expression.

#### Antisense Oligo Transfection and Full-Length cDNA Transfection

For antisense oligo transfection, a stretch of 23-bp-long DNA sequences of tyrosine protein kinase ABL2 (*ABL2*) cDNA from Genebank (accession no. M35296) was chosen for synthesizing both sense and antisense oligos (sense, agagcagggatggggcagcaggt; antisense, acctgctgccccatccctgctct), which cover the ATG start codon of the gene. Both oligos are incorporated with phosphorothioate to increase their intracellular stability. In each transfection, 0.55  $\mu$ M oligo and 10  $\mu$ g of lipofectamine in 1 ml of serum-free 1640 medium were applied to the growing cells in a T-25 flask containing  $0.5 \times 10^6$  to  $1 \times 10^6$  cells. Triplicate flasks for both sense and antisense oligo transfection were used. After incubation at 37°C for 8 hours, 4 ml of complete RPMI 1640 medium was added and cells were incubated for another 48 hours, and then trypsinized and counted. In cDNA transfection studies, the full-length cDNA of the *Hint/PKCI-1* or *TRP-1* gene, amplified by RT-PCR from cell line NCI-H522, were cloned into the *XhoI* recognition site of the pLXSN vector (Clontech). Primer sequences used for cloning the full-length cDNA of the *Hint/PKCI-1* gene were: upstream, acgtctcgaggcgagatggcagatgagattg; downstream, atgtctcgagacgtgcttaaccaggaggccaatg. Primer sequences for cloning full-length cDNA of *TRP-1* gene were: upstream, gaccctcgagcagaatgagtgtctctaaactcc; downstream, gctactcgagtagggcattgttagaccacagac. The insert and its orientation from recombinant clones were confirmed by restriction analysis and DNA sequencing. Five micrograms of plasmid DNA containing either forward-oriented or reverse-oriented



**Figure 3.** RT-PCR confirmation of the alterations in gene expression identified by cDNA array analysis in NCI-H522 cell line. *ERK6*, *ABL2*, *Myt1*, *RGC1*, *RAD21H*, *KRS2*, *hEGR1*, *ITGB8*, and *ARAF1* genes are downregulated, whereas *CKS1*, *CKS2*, *S100-A4*, *TXBP151*, *CAF1*, *HP1*, and *TIMP-1* genes are upregulated by 1  $\mu$ M 5-aza-dC treatment for 4 days.

**Table 1.** Alterations in Gene Expression in NCI-H522 Cells Treated by 5-Aza-dC.

Accession	Gene or Protein Name (Symbol)	Primers User for RT-PCR
<i>Genes downregulated by 5-aza-dC</i>		
L07868	ERBB4 receptor tyrosine kinase	cttcaagcattggataatocccaatatcac/agcttaccacacagattatocgggtgtctgta
L24038	A-raf protooncogene homolog 1 (ARAF1)	tcaaagtataacctgcocacaagaacac/cttcaaggaoctcgacaatgagctc
AF010310	p53-induced protein	gtgcgcagatcggtatgaggacc/gggtgocctcatgaggctgctg
X79483	Extracellular signal-regulated kinase 6 (ERK6)	agctgaagatcctggactcgggoc/gggagccctcatgtagttcttgg
M35296	Tyrosine protein kinase ABL2 (ABL2)	aggtagctgaggagcttgggagag/ttctgttcgaggcagtgctgggg
AF014118	Membrane-associated kinase 1 (MYT1)	gggocattggctcctcaggagag/ccagggttcacagtgtgctgcag
D87119	Cancellous bone osteoblast	ccagctgggtgcccggacgtcaac/ccatgctacgtggctcagtcagctc
U48296	pTPCAAX1 nuclear tyrosine phosphatase	cctgggtgttattgctgttctatgc/tgaccgttggaaatttgaacgcag
X78817	rho-GAP hematopoietic protein C 1 (RGC1)	ctgcttagcctggcctagtgtaacg/gtctcaatggctgtcgtgcagc
U60207	Serine/threonine protein kinase KRS2 (KRS2)	ggcttgctcatgtttgttagccag/ctcaactaggagctctgttctctgg
D38551	RAD21 ( <i>S. pombe</i> ) homolog (RAD21H)	gtatcaatgggtgggctgtagtgc/gggctcaattgtctgtatccaac
U43431	DNA topoisomerase III $\alpha$	gagaccacagtgagatcgacatcg/gcatocgtaccaatgccatgctct
X52541	Early growth response protein 1 (hEGR1)	tggctccaggttccatgatcccg/ggcaagcgtaaggcgcttctgtggg
M73780	Integrin precursor (ITGB8)	gtgcccaatgacggaaactgtcatctg/cattgctgcctacttctgcatocctc
<i>Genes upregulated by 5-aza-dC in NCI-H522 cells</i>		
M63618	Bullous pemphigoid antigen 1	tgttcagggttattggctgactgctag/gagtgaacctgtggctctatcaacoc
M73980	Notch protein homolog 1	cgcaggctcagcggtatocac/gtactgggtgtgggtctgccagc
M14505	Cyclin-dependent kinase 4 (CDK4)	cttcocatcagcacagttcgtgagg/cttgactgttcaocactgtcacc
X54941	Cyclin-dependent kinase regulatory subunit 1 (CKS1)	catgtcgcacaaacaaattactattcgg/agatgtgaggttctgttctatggatc
X54942	Cyclin-dependent kinase regulatory subunit 2 (CKS2)	agtctocggcgagttgttgcctg/gactctgttgacaccaagctcc
U51004	Hint protein; protein kinase C inhibitor 1 (Hint/PKCI-1)	gaagatcatocgcaaggaaataaccag/cttattcaggccagatcagcagc
M80563	S100 calcium-binding protein A4(S100-A4)	gtttgatcctgactgctgtcatgg/gcatcaagcacgtgtctgaaggagc
U46461	Dishevelled 1 (DVL1)	tcaccatcgccaatgcccgtcatcg/tggagccactgttgaggttcaggg
U33821	TAXI-binding protein 1 51 (TXBP151)	tcctgatctccaagtcaacatttacg/caaacacctgctggtcatagtcagg
U56390	Caspase-9 (CASP9)	agctggagccatattctagtgttgcgc/ggtgcaagataaggcagggtgaggg
M15796	Proliferating cyclic nuclear antigen (PCNA)	gaagggtgttgaggcactcaaggag/ggtgcttcaatactagcgccaagg
L07515	Heterochromatin protein homolog 1 (HP1)	gggcagacgttagcgtgagtgatc/atattccactgttccctaaocacgc
X74262	Chromatin assembly factor 1 p48 subunit (CAF1)	agagtgcaaoaccagacttgctgtc/ocaggaaacattcttactactgccg
X03124	Tissue metalloproteinase inhibitor 1 (TIMP1)	tgcaattcccagctcgtcatcagggc/agaatacctcgtcgcggttggg
U36223	Fibroblast growth factor 8	ccaacaagcgcatcaacgccatgg/cagccctgactgtgcatctgc
X51420	Tyrosinase-related protein 1 (TRP-1)	ctgcacgatgactgatgggatcc/cttcaagcactgagcgacatcctc

full-length cDNA in the expression vector was used in each lipotransfection. After 48 hours of incubation, cells were selected by 200  $\mu$ g/ml G418 for about 20 days. The mixture of G418-resistant clones was then collected.

#### Nude Mice Tumorigenicity Assay

Exponentially growing cells collected from stable transfection were harvested and resuspended in serum-free medium. After washing twice with serum-free medium,  $2 \times 10^6$  cells were inoculated subcutaneously at the proximal dorsal midline of 4- to 6-week-old female Balb/c athymic nude mice (Jackson Laboratories, Inc., Bar Harbor, ME). Tumor size was measured in two dimensions twice a week. Tumor tissue generated at the injection site was dissected at the end of the assay, fixed in 4% paraformaldehyde buffer (pH 7.0), and embedded in paraffin for pathologic examination. The weight of tumor dissected was measured and a *t*-test was used to compare the numbers obtained from different injection groups. Tumor growth was typically ob-

served for 90 days. Following the institute's guidance on animal care, nude mice with steadily growing tumors reaching a size of  $10-15 \times 10-15 \text{ mm}^2$  were sacrificed before the end of observation period. Histopathologic examination was performed on the specimens stained with hematoxylin and eosin.

## Results

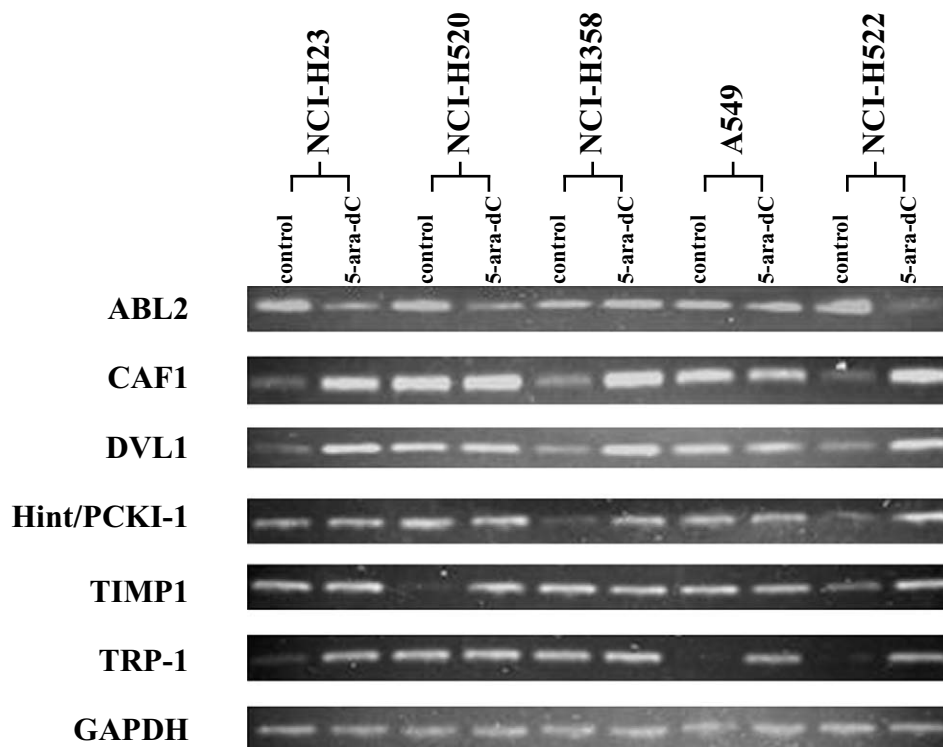
### 5-Aza-dC Induces Cell Apoptosis and Cell Cycle Arrest in NCI-H522 Cells

To find an appropriate cell line whose response to 5-aza-dC is derived mainly from drug-induced DNA demethylation, five NSCLC cell lines were screened by 1  $\mu$ M 5-aza-dC treatment for 4 days. In all lines, 5-aza-dC caused a significant inhibition of cell growth compared to the untreated cell lines. However, all but the NCI-H522 cell line exhibited profound cytotoxicity as demonstrated by dramatic cell death

**Table 2.** Suppression of *In Vivo* Tumorigenicity by Hint/PKCI-1 in NCI-H358 Cell Line.

Cell Lines/Transfection	Latency (days)	Tumor Weight (g)	Number of Tumors/Mice
NCI-H358/vector	18–24	0.310 $\pm$ 0.080	3/3
NCI-H358/Hint/PKCI-1	18–24	0.120 $\pm$ 0.025 ( $P < .01$ )	3/3
NCI-H358/vector*	17–22	0.350 $\pm$ 0.080	3/3
NCI-H358/Hint/PKCI-1*	19–24	0.130 $\pm$ 0.020 ( $P < .01$ )	3/3

\*Numbers were obtained from the repeated experiments.



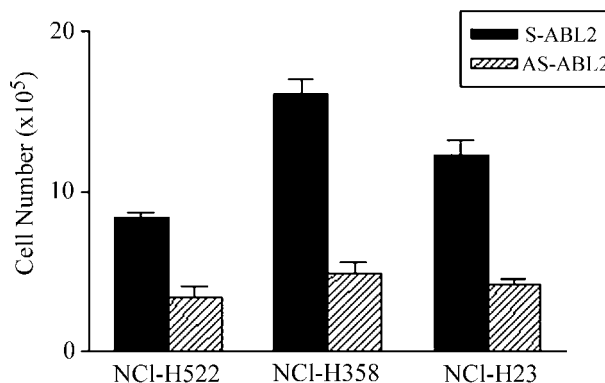
**Figure 4.** RT-PCR to detect the expression of candidate genes in five human NSCLC cells lines treated by 1  $\mu$ M 5-aza-dC. Downregulation of the ABL2 and upregulation of the CAF1, DVL1, Hint/PCKI-1, TIMP-1, and TRP-1 genes were found in three, three, three, two, two, and three of five cell lines, respectively. The expression of GAPDH was used as quantitative standard.

and detachment from culturing flask after 5-aza-dC treatment. 5-Aza-dC-induced cell growth inhibition in NCI-H522 cells was further characterized by cell proliferation assay, TUNEL assay, and cell cycle analysis. The cell proliferation assay showed that a slowdown on cell growth started at 48 hours after 1  $\mu$ M 5-aza-dC treatment, followed by nearly a complete cessation of cell proliferation (Figure 1A). The TUNEL assay showed that apoptotic cells started to increase on the third day after 5-aza-dC treatment and reached 13% on the fifth day postdemethylation treatment compared with 2% in untreated cells (Figure 1B). The flow cytometry assay indicated that the percentage of cells in S-phase was dramatically increased on the fifth day of 1  $\mu$ M 5-aza-dC treatment (Figure 1C). These data indicated that the 5-aza-dC-induced cell growth inhibition was partially attributable to cell cycle arrest and apoptosis.

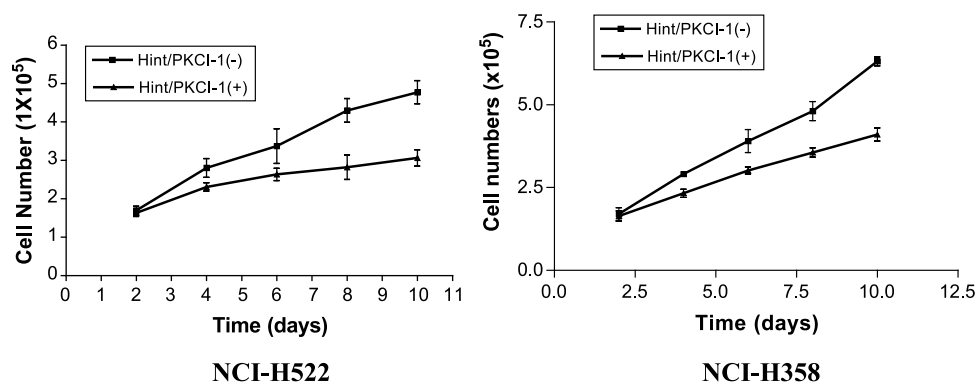
#### Alterations of mRNA Expression Were Identified in 5-Aza-dC-Treated NCI-H522 Cells

To avoid ambiguity caused by potential 5-aza-dC-induced cytotoxicity during gene expression analysis, only the NCI-H522 cell line was chosen for cDNA array analysis. To identify genes undergoing changes in mRNA expression after 5-aza-dC treatment, a membrane cDNA array analysis covering 1176 genes was employed. The cDNA array analysis was performed by using a comparison between 4 days of 1  $\mu$ M 5-aza-dC-treated and untreated cells. Quantitative cDNA array data were determined by the ratio of the adjusted intensity of each gene with that of the average of all house-

keeping genes provided in the same cDNA array, including ubiquitin and GAPDH. Under our experimental conditions, the average intensity values of all housekeeping genes were consistent with that of ubiquitin and GAPDH between treated and untreated cells. The genes with an absolute adjusted intensity over 500 and with a ratio difference over five-fold between treated and untreated samples were chosen for further analysis. A representative cDNA array analysis is shown in Figure 2. Alteration of mRNA expression of a total of 73 genes was determined by these criteria from



**Figure 5.** Cell number counting after antisense oligo transfection for ABL2 gene in human NSCLC cell lines showing downregulation of ABL2 gene after 5-aza-dC treatment. Significant differences in cell numbers between sense oligo-transfected (S-ABL2) and antisense oligo-transfected (AS-ABL2) cells were observed in NCI-H522, NCI-H358, and NCI-H23 cells.



**Figure 6.** Cell proliferation assays for NCI-H522 and NCI-H358 cell lines transfected with *Hint/PKCI-1* cDNA. Slower growth was observed in both cell lines transfected with forward-oriented cDNA, indicated as *Hint/PKCI-1(+)*, in pLXSN expression vector as compared with that transfected with reverse-oriented cDNA, labeled as *Hint/PKCI-1(-)*.

repeated experiments. After further confirmation by RT-PCR, 30 genes, either downregulated or upregulated in 5-aza-dC-treated cells, were considered as candidate genes for further characterization (Table 1). Examples such as downregulation of *ABL2*, membrane-associated protein (*Myt-1*), Rho-Gap hematopoietic protein C1 (*RGC1*), and early growth response protein 1 (*hEGR1*) genes; and upregulation of *Hint/PKCI-1*, dishevelled 1 (*DVL1*), chromatin assembly factor 1 (*CAF1*), tissue metalloproteinase inhibitor 1 (*TIMP-1*), and tyrosinase-related protein 1 (*TRP-1*) genes are shown in Figure 3.

#### Genes Showing Alteration in mRNA Expression in Multiple 5-Aza-dC-Treated NSCLC Cell Lines

To determine which genes showed recurrent changes in gene expression in multiple cell lines, the detection of mRNA expression of all 30 candidate genes was expanded to NCI-H23, NCI-H358, HCI-H520, and A549 cell lines treated similarly with 5-aza-dC. All RNA samples used in these detections were equally quantified relative to the expression of the *GAPDH* gene. The downregulation of *ABL2* gene and the upregulation of *Hint/PKCI-1*, *CAF1*, *DVL1*, *TIMP-1*, and *TRP-1* genes were observed in three, two, three, three, two, and three of five cell lines treated with 5-aza-dC, respectively (Figure 4).

#### Upregulation of *Hint/PKCI-1* Gene Was Directly Associated with Demethylation-Induced Cell Growth Inhibition

To determine the association of the alterations in gene expression of the candidate genes with DNA demethylation-induced cell growth inhibition, *ABL2*, *Hint/PKCI-1*, and *TRP-1* were selected for functional analysis. The antisense oligo transfection for the *ABL2* gene and full-length cDNA transfection for the *Hint/PKCI-1* and *TRP-1* genes were performed in cell lines that showed alterations in mRNA expression of these genes after DNA demethylation treatment. A significant cell growth inhibition was observed in *ABL2* antisense oligo-transfected NCI-H522, NCI-H23, and NCI-H358 cell lines compared with each sense oligo-transfected cell line (Figure 5). No difference in *ABL2* mRNA expression was observed between antisense oligo and sense oligo-trans-

fected cells (data not shown). The attempt to detect the change in protein level after antisense oligo transfection failed due to the lack of antibody against *ABL2* protein. In *Hint/PKCI-1* full-length cDNA transfection study, a marked cell growth inhibition in cell proliferation assays using 2% serum was observed in both NCI-H522 and NCI-H358 cell lines (Figure 6). However, no obvious cell growth inhibition was observed in *TRP-1* cDNA-transfected A549, NCI-H23, and NCI-H522 cell lines. The expression of the transfected gene was detected by RT-PCR only in cells transfected with forward-oriented cDNA in expression vector (data not shown).

#### *Hint/PKCI-1* Gene Reduced In Vivo Tumorigenicity of NCI-H358 Cells

To assess the suppressive effect of the *Hint/PKCI-1* gene on tumorigenic potential of both NCI-H522 and NCI-H358 cells, we tested the tumorigenicity of two stably transfected cell lines in nude mice. Animals that received NCI-H358 cells transfected with either pLXSN-*Hint/PKCI-1(+)* or pLXSN alone developed tumors during the first 4 weeks of the experiment. However, tumors from pLXSN alone continued to grow progressively during the observation period, whereas tumors from *Hint/PKCI-1(+)* transfection exhibited a significantly slower growth over the entire period of the examination. The difference in tumor size between two injection groups was very significant ( $P < .01$ ). Histopathologic analysis of the tumors was consistent with lung adenocarcinoma. The NCI-H522 cell line, although derived from lung adenocarcinoma, did not produce tumors in nude mice. The nude mice *in vivo* tumorigenicity assays were repeated twice and the results are summarized in Table 2.

#### Discussion

Our experiments show that 5-aza-dC can significantly induce the inhibition of *in vitro* growth of human NSCLC cell lines through DNA demethylation-induced changes in gene expression. The observations of cell apoptosis and cell cycle arrest in S-phase in 5-aza-dC-treated NCI-H522 cells, two contributing factors to drug-induced cell growth inhibition, are consistent with the general understanding of the

consequence of 5-aza-dC–induced DNA demethylation. These changes in tumor cell phenotypes are believed to be based on the induction of alterations in gene expression. During the course of growth inhibition of NCI-H522 cells, the mRNA expression of 16 genes was upregulated, whereas that of 14 genes was downregulated in over 1000 genes tested in cDNA array analysis. These genes belong to different categories in terms of regulation of cell functions. The *CKS1* gene, for example, is involved in the regulation of cell cycle [26]; the *CASP9* gene is implicated in the regulation of cell apoptosis [27]; and the *ERK6* gene may contribute to cell transformation [28]. However, the genes with recurrent alteration in other 5-aza-dC–treated cell lines are also more likely involved in the regulation of cell growth. The expression of the *ABL2*, *Hint/PKCI-1*, *DVL1*, *CAF1*, *TIMP-1*, and *TRP-1* genes was found to be altered in more than two NSCLC cell lines used in this study, implying a contribution of these genes to cell growth inhibition in multiple NSCLCs.

Our data strongly suggest that the *Hint/PKCI-1* gene functions as a negative regulator of tumor cell growth because 5-aza-dC–induced upregulation of the *Hint/PKCI-1* was found in two of five NSCLC cell lines. Overexpression of the *Hint/PKCI-1* by cDNA transfection resulted in *in vitro* cell growth inhibition of these two NSCLC cell lines. In addition, stable transfection of *Hint/PKCI-1* cDNA significantly reduced *in vivo* tumorigenicity of NCI-H358 cells. *Hint/PKCI-1* is the only other characterized human histidine triad (HIT) nucleotide-binding protein in addition to tumor-suppressor gene *FHIT* [29], which is a frequent target of genomic deletion and aberrant DNA methylation in human lung cancer as well as other kinds of cancer [30–32]. Like *FHIT*, the *Hint/PKCI-1* gene is also involved in nucleotide metabolism by binding nucleotide molecules and hydrolyzing ADP *in vitro* [33]. However, the involvement of the *Hint/PKCI-1* gene in carcinogenesis has not been investigated. The *Hint/PKCI-1* gene is localized at chromosome 7q21–22, a region of genomic deletion reported in radon-induced rat lung cancer [34] and in more invasive human NSCLCs [35]. In addition, microcell transfer studies suggested the existence of a metastasis-suppressor gene in this region [36]. Thus, *Hint/PKCI-1* might represent a negative regulator of tumor cell properties in this region. The examination of *Hint/PKCI-1* expression in primary tumors would provide more convincing evidence on the role of this gene in NSCLCs. In addition, it would be interesting to see whether gene expression of both *Hint/PKCI-1* and *FHIT* is altered in NSCLCs.

Downregulation of *ABL2* was detected in three of five NSCLC cell lines treated with 5-aza-dC. In addition, antisense oligo transfection against *ABL2* caused a significant *in vitro* cell growth inhibition in these three cell lines. However, in the absence of evidence showing that antisense strategy resulted in the decrease of protein expression of *ABL2*, one cannot speculate that this protooncogene is involved in the pathogenesis of NSCLC.

Functional exploration of the *ABL2*, *Hint/PKCI-1*, and *TRP-1* genes in this study is part of identifying genes in DNA demethylation–treated NSCLC cells that may be relevant in the pathogenesis of lung cancer. The other candidate

gene selected for future functional characterizations is *CAF1*, which was first found to be a retinoblastoma protein (Rb)–binding protein and a negative regulator of Ras in yeast [37]. It was later found to be a component of the histone deacetylase complex recruited by Rb and to mediate transcriptional repression of E2F1 [38].

In summary, our observations demonstrate that 5-aza-dC can significantly induce the growth inhibition of human lung cancer cells by DNA demethylation and the consequent changes in mRNA expression of multiple genes. Also, our approach of characterizing the variation of gene expression during the transition of DNA methylation thus is apparently efficient in identifying new cancer-related genes. Promoter methylation status will be characterized to determine whether the changes in gene expression of these genes are caused by their own aberrant promoter methylation, or are derived from the regulation of other genes, which are primarily regulated by DNA demethylation. These studies should shed more insight into the functional understanding of the candidate genes identified in this study.

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